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The pendulum of the Ku-Ku clock

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ABSTRACT

Canonical DNA non-homologous end-joining (c-NHEJ) and homologous recombination (HR), the two major DNA double-strand break (DSB) repair pathways, have long been depicted as competitors, fighting a race to rejoin DSBs. In human cells, Ku, an upstream component of NHEJ, is highly abundant and has exquisite end-binding capacity. Emerging evidence has suggested that Ku is the first protein binding most, if not all, DSBs, and creates a block to resection. Although most c-NHEJ proceeds without resection, recent studies have provided strong evidence for a process of resection-dependent c-NHEJ, that repairs a subset of DSBs. HR also repairs a subset of two-ended DSBs in G2 phase and processes one-ended DSBs that arise following replication fork stalling or collapse to promote replication restart. HR also necessitates end-resection. This raises the question of how end-resection takes place despite Ku's avid end-binding capacity. Insight into this enigma has been gained from the analysis of DSBs generated by Spo11 or TOP2, which create protein-bridged DSBs. The progression of repair by HR or NHEJ requires removal of the end-blocking lesions. The MRE11-RAD50-NBS1 (MRN) complex, CtIP and EXO1 play critical roles in this process. Here, we review our current understanding of how resection arises at lesions blocked by covalently bound Spo11 or TOP2 or following Ku binding, which effectively creates a distinct resection-blocking lesion due to its avid end-binding activity and abundance. Our review reveals that Ku plays an active role in determining pathway choice and exposes similarities yet distinctions in the progression of resection that is suited to the optimal repair pathway choice.

1. Introduction

DNA double-strand breaks (DSBs) can arise from external or endogenously arising DNA damaging agents (e.g. ionising radiation (IR) or reactive oxygen species), from problems during processes including replication and transcription or during developmental processes such as V(D)J recombination or meiosis. DSBs can have distinct end configurations and be one-ended or two-ended. DSBs directly induced by DNA damaging agents are normally two-ended. Accurate repair of such ends necessitates that the correct ends are rejoined without junctional sequence loss. Most ends arising from DNA damaging agents require end-processing. However, cells are equipped with a plethora of end-processing enzymes which, provided sequence information is not lost from both strands, create ligatable ends. High linear energy transfer (LET) radiation, and to a lesser extent low LET radiation such as X-rays, can result in sequence loss [1]. DSB ends can also be blocked for repair, for example by covalently bound proteins, such as topoisomerase I or II (TOP1 or 2) [2]. Intriguingly, during V(D)J recombination and some retrotransposition, hairpin-ended DSBs are generated [3]. In distinction, one-ended or single-ended DSBs (seDSBs) can arise following replication fork collapse [4]. In this situation, accurate repair necessitates re-instating the replication fork; rejoining using another DSB end will cause genomic instability, described as "toxic" DSB repair.

There are two major DSB repair pathways and several "back-up" pathways that promote rejoining at the cost of lowered fidelity [5]. Canonical non-homologous end-joining (c-NHEJ) is the major DSB repair pathway. It represents a relatively simple process with the highly abundant Ku heterodimer binding to DSB ends followed by recruitment of the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to generate the DNA-PK complex. Ku-bound DNA ends can block

Abbreviations: DSBs, DNA double-strand breaks; IR, ionising radiation; high LET radiation, high linear energy transfer radiation; TOP1/2, topoisomerase I/II; seDSBs, single-ended DSBs; c-NHEJ, canonical non-homologous end-joining; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; HR, homologous recombination; alt-NHEJ, alternative NHEJ; SSA, single-strand annealing; Exo1, exonuclease 1; SSBs, single-strand breaks; CPT, camptothecin; ss-DNA, single-stranded DNA; MRN, MRE11-RAD50-NBS1; PLK3, polo-like kinase 3; TDP2, 5′-tyrosyl DNA phosphodiesterase 2

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exonucleolytic activity [6]. DNA-PK, as well as filament formation involving XRCC4 and XLF, may promote end-synapsis and end-stability. Finally, a complex involving XRCC4-DNA ligase IV promotes ligation. Although NHEJ is highly efficient and mutants lacking NHEJ proteins are extremely radiosensitive, it does not have the capacity to reconstitute sequence information lost on both strands. Another limitation of NHEJ is that, since it requires little or no junctional sequence homology, it has the potential to rejoin the wrong DSB ends if synapsis is not maintained.

Homologous recombination (HR) is another DSB repair pathway, which is the antithesis in terms of complexity compared to the elegantly simple NHEJ process [5]. In brief, HR employs an undamaged sister chromatid to promote repair, thereby providing the capacity to reconstitute lost sequence information using the undamaged strand as a template. The first step of HR involves resection of the DSB end to generate a 3' overhang. Resection is a complex process involving multiple nucleases and, in a less defined way, BRCA1 [7,8]. BRCA2 in combination with PALB2 then promotes RPA replacement by RAD51. RAD51-bound DNA invades the template strand generating a D-loop and heteroduplex DNA. HR is completed by DNA repair synthesis of the broken strand and resolution of the heteroduplex DNA either with or without cross-over formation. It is noteworthy that not only does HR have the ability to accurately reconstitute junctional sequence information but, due to the requirement for substantial homology, it has a lower tendency to rejoin incorrect DNA ends. However, by necessitating a sister chromatid as a template, HR only occurs in late S/G2 phase. Perhaps surprisingly, the major role of HR is not to rejoin two-ended DSBs but rather to restore an active replication fork following replication fork collapse and to preclude "toxic" NHEJ at seDSBs [4]. However, there is increasing evidence that HR may have a role in repairing specific DSBs such as those at transcriptionally active regions, where accurate rejoining is important [9]. HR also predominates during meiosis, where cross-over formation is exploited to promote genetic variability.

Alternative NHEJ (alt-NHEJ) and single-strand annealing (SSA) are two "back-up" pathways that exploit microhomology to aid rejoining at the expense of reduced fidelity due to junctional deletions [10,11]. Neither pathway appears to arise frequently in normal human cells although they function in situations where NHEJ or HR cannot progress, including in cancer cells.

A prevailing question is how the choice between these DSB repair pathways is regulated and influenced by the nature of the DSB. Although multiple early studies discussed and examined competitive binding between HR proteins and Ku at DSBs, more recent studies are consistent with the notion that, due to its high abundance and strong end-binding capability, Ku wins the fight and predominates in binding most DSBs. Consequently, progression of HR or back-up pathways necessitates the eviction of Ku or the relief of its barrier to resection. Given this, the question becomes: how do resection-dependent pathways ensue in the face of Ku's abundant and prolific end-binding capacity?

Paradoxically, an important clue in unearthing this question emerged from a study deciphering early steps during meiosis using *S. cerevisiae*, where Ku is unable to bind due to the presence of the covalently bound enzyme that created the DSBs [12,13]. During meiosis, DSBs are generated by Spo11, a TOP2-like enzyme, which creates protein bridged DSBs [14]. In contrast to Ku, Spo11 is covalently bound at DSB ends. Thus, to enable resection and the progression of HR during meiosis, Spo11 must be removed. Insight, which has been supported by subsequent biochemical studies, revealed the role of the bifunctional endo/exonuclease activity of Mre11 together with exonuclease I (Exo1) [13,15]. Strikingly, following the formation of Spo11-linked 5'-DNA termini, Mre11 endonucleolytically cuts the 5' strand up to 300 nucleotides from the terminus, with subsequent resection ensuing in a bidirectional manner with Exo1 resecting 5'–3' away from the DSB and Mre11 exonuclease progressing 3'–5' towards the end (Fig. 1). This process represents an efficient means to remove a protein-block at the DSB end. Significantly, although Ku binds DSBs non-covalently, its high abundance likely results in rapid rebinding when lost from a non-resected end, creating in effect a form of protein-blocked DSB end. Garcia et al. insightfully proposed that this type of Mre11-catalysed resection might be a general feature of DSB repair pathways [13]. Variations to this process depending on the precise situation and DSB nature are central to our discussion below.

Here, we review recent insight into how resection proceeds at DSB ends following Ku binding. Since the optimal pathway is strongly influenced by cell cycle stage, we will consider the processes operating in S, G2 and finally G1 phases, and discuss the commonalities and distinctions between the processes.

2. Resection occurs despite Ku's preponderance in S and G2 phase

2.1. HR is the pathway of choice for one-ended DSBs arising at the replication fork

HR can repair two-ended DSBs but, as mentioned above, its arguably more important role lies at the replication fork where it promotes the recovery of collapsed forks via a function on seDSBs. Replication forks transiently stall at DNA lesions when the progression of the polymerases or helicases is stopped. The error-free recovery of such stalled forks by HR involves the process of fork reversal leading to the formation of a chicken-foot structure with a double-stranded DNA end formed from the annealing of the newly synthesized DNA [16]. Prolonged replication fork stalling can lead to the disassembly of replication proteins, a process called replication fork collapse. The repair of collapsed replication forks typically involves the generation of seDSBs [17]. SeDSBs can also arise when replication forks encounter unrepaired single-strand breaks (SSBs) either formed as repair intermediates during base excision repair or as a result of topoisomerase cleavage [18,19].

The DNA ends arising at a reversed fork, following replication fork collapse or from replication encounters at unrepaired SSBs, typically undergo resection and HR to re-initiate replication. Although NHEJ of such end-structures would be detrimental, given Ku's abundance, it is perhaps not surprising that Ku binds to such ends [20–23]. This raises the question of how resection is executed with Ku 'blocking' the end and forces a comparison with the resection of Spo11-bound DSBs during meiosis. Recent work from the Calsou laboratory has investigated the repair of seDSBs induced by the TOP1 inhibitor camptothecin (CPT) in human cells [24]. Remarkably, they observed that RPA was loaded onto single-stranded DNA (ss-DNA) and subsequently phosphorylated in a DNA-PK dependent manner, suggesting that Ku and RPA loading takes place with Ku bound to DSB ends. Further analysis revealed that depletion of CtIP, a central resection factor that functions to promote MRE11 nuclease activity, or expression of a nuclease deficient MRE11 mutant abolished the formation of RPA foci [25–27] (Fig. 2). Moreover, expression of an MRE11 mutant deficient for its exonuclease activity but retaining its endonuclease function, allowed some RPA foci formation but failed to clear Ku from the DSB ends. Collectively, this work suggested that, akin to the situation in meiosis, CtIP together with MRE11 endonuclease initiate resection internally to the Ku-bound DSB site. MRE11 then functions as an exonuclease, promoting 3′–5′ resection towards the break end to generate a structure with Ku bound at the end. Finally, use of a mutant defective in CtIP nuclease activity provided evidence that Ku is released via CtIP's endonuclease activity. This CtIP nuclease activity requires its phosphorylation by ATM, highlighting a role for ATM kinase in regulating Ku removal [28]. Concomitant with MRE11 exonuclease function, EXO1 enlarges the ss-DNA region by promoting 5′–3′ resection away from the break site.

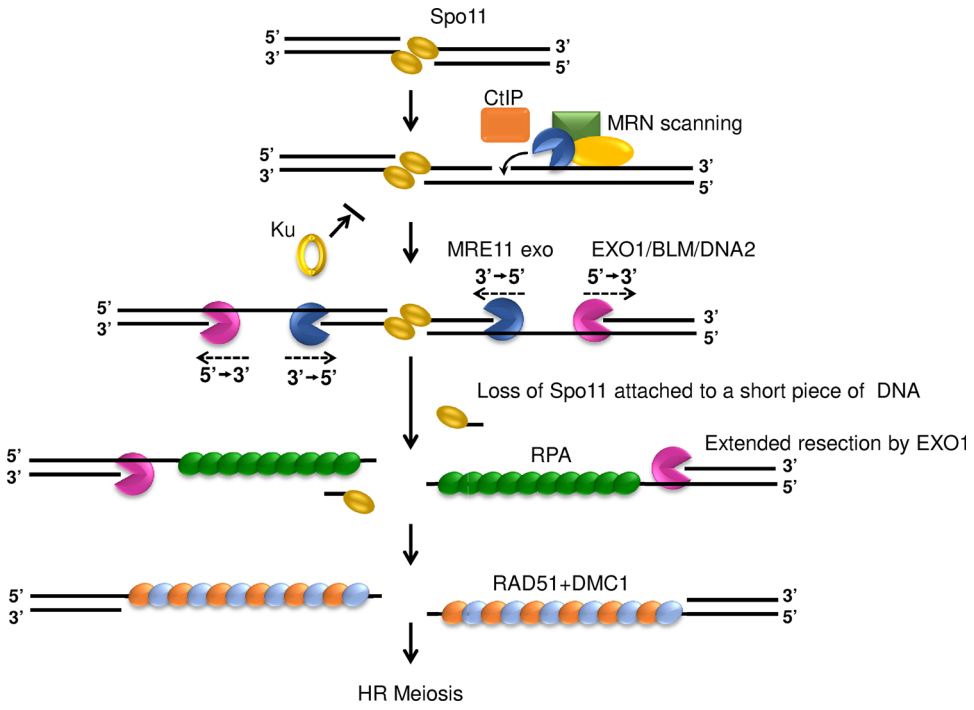

Fig. 1. Progression of HR during meiosis. Spo11 creates protein-bridged DSBs as the initiating step for homologous recombination during meiosis. Resection is initiated by MRE11 endonuclease activity, which is activated by CtIP. Bidirectional resection then ensues by MRE11 exonuclease and EXO1/BLM/DNA2, which have the polarities shown. The final step resulting in Spo11 removal remains unclear. After denaturation Spo11 is detectable attached to a short piece of DNA. Upstream steps in this process have been revealed using S. cerevisiae and have not yet been substantiated using higher organisms. We show the nomenclature for the nucleases for human proteins to allow comparison with subsequent figures. We depict RAD51 and DMC1 as forming mixed filaments although there is also evidence for the recombinases forming distinct filaments.

2.2. NHEJ and HR can repair two-ended DSBs arising in G2

Given the existence of a sister chromatid in G2 phase, it was perhaps expected that HR would dominate over NHEJ in repairing two-ended DSBs in G2. However, early work indicated that in normal G2 cells a substantial fraction of IR-induced DSBs are repaired by NHEJ, a notion consolidated in subsequent studies [29–31]. Fundamental to these studies is a cell cycle specific approach which allows the investigation of cells damaged in G2 and subsequently maintained in G2 during repair, a strategy that avoids the analysis of replication-associated DSBs which are predominantly repaired by HR [32]. Detailed kinetic analyses revealed that repair of IR-induced DSBs in G2 ensues with biphasic kinetics: a fast process representing NHEJ repairs 70–80% of the breaks within the first 2–3 h while HR proceeds with substantially slower kinetics and repairs the remainder of the induced lesions [30]. The slow component in this study involved ATM's function to phosphorylate KAP-1, which was shown previously to promote repair of heterochromatic breaks [33], suggesting that HR primarily repairs IR-induced DSBs localizing to heterochromatin or undergoing heterochromatization during repair [34].

Another factor influencing the choice between NHEJ and HR for repairing two-ended DSBs is lesion complexity. While low LET X- or γ-radiation induce DSBs with limited end complexity, high LET radiation such as α-particles and heavy ions cause DSBs in close proximity with each other and with other DNA lesion types [1,35,36]. Such complex DSBs undergo significantly slower repair than chemically less severe DSBs and, importantly, require HR for their repair in G2 phase [31].

In contrast to the strict dependency of complex DSBs on HR for repair in G2, simple X- or γ-radiation induced DSBs, as well as DSBs induced by radiomimetic drugs, appear to be repairable by either NHEJ or HR. In line with this notion, inactivation of the initiation of resection by CtIP depletion prevented repair by HR but allowed the slowly repairing DSBs to be handled by NHEJ, while inactivation of a downstream HR factor such as BRCA2 caused a repair defect [31]. This suggests that Ku binds to DSBs undergoing slow HR repair and indeed promotes NHEJ if HR is not initiated, a situation reminiscent of the repair of CPT-induced seDSBs in S phase [24]. However, whereas repair of seDSBs by NHEJ leads to structural chromosomal aberrations and cell death, NHEJ of slowly repairing DSBs in G2 may not be detrimental.

A model explaining the finding that both NHEJ and HR can handle slowly repairing IR-induced DSBs in G2 is that Ku remains bound to the break end during resection until a DSB end structure is generated which prevents NHEJ and commits repair to HR (Fig. 3). A substantial stretch of ss-DNA likely represents a structure precluding NHEJ, predicting that the function of the resection exonucleases commits repair of two-ended DSBs to HR. In line with this model, Shibata et al. [37] observed that inhibiting MRE11's exonuclease function together with EXO1 prevented HR but allowed repair by NHEJ, while inactivation of either exonuclease alone prevented HR but precluded a switch to NHEJ. Thus, both exonucleases are required for HR but either alone generates a stretch of ss-DNA that provides a commitment step for HR. Moreover, inhibition of MRE11's endonuclease function alone was sufficient to prevent HR but allowed a switch to NHEJ, suggesting that MRE11 functions as an endonuclease upstream of the exonucleases and promotes, together with CtIP, the initiation of resection [37]. This finding taken together with a consideration of the opposite polarities of MRE11 exonuclease and EXO1 strongly suggests that the same model proposed for the repair of Spo11-induced DSBs in meiosis and CPT-induced DSBs in S phase functions to regulate the switch from NHEJ to HR in G2 phase. Specific features of resection in S and G2 are highlighted in Figs. 2 and 3, and will be discussed in further detail below.

3. A sub-pathway of NHEJ involves resection and repairs 'difficult' DSBs arising in G1

3.1. A bit of an historical account

The prevailing dogma has been that DSB repair in G1 occurs by NHEJ without resection since a sister chromosome is not present to support HR and RPA foci are usually not observed in G1 cells after X- or γ-irradiation. Indeed, studies have shown that NHEJ is the major, and likely the sole, DSB repair pathway in G1 [29,38,39]. Despite this exclusivity of NHEJ in G1 phase, a kinetic analysis revealed the existence of two distinct repair components. Similar to the situation in G2, 70–80% of DSBs are repaired within 2–3 h with the remainder being repaired with slow kinetics [39]. This slow repair component involves the classical NHEJ factors, DNA-PK and DNA ligase IV, and additionally the nuclease Artemis and proteins that localize to γH2AX foci, including

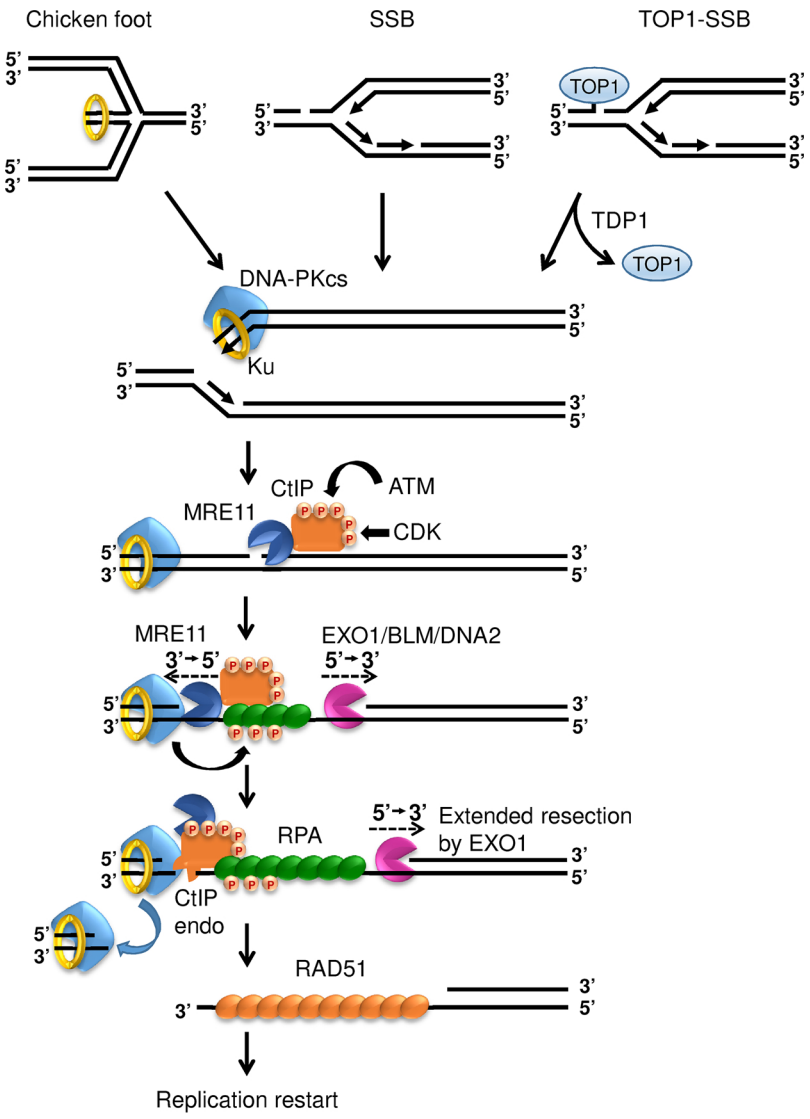

Fig. 2. Progression of resection at seDSBs arising following CPT treatment.

A double-stranded DNA end arises following fork stalling and reversal, and promotes fork recovery. Replication fork collapse leads to a seDSB, which can also arise when replication forks encounter SSBs formed during, e.g., base excision repair or from topoisomerase cleavage (TOP1-SSB). Our figure is based primarily on a seDSB arising following treatment with CPT. We depict the covalently bound TOP1 blockage being removed by TDP1. Ku then binds to the seDSB and must be removed to allow resection and HR. The initiating steps are similar to those shown in Fig. 1. For the depicted process, it has been argued that Ku is removed following CtIP endonuclease activity.

ATM, 53BP1 and the MRE11-RAD50-NBS1 (MRN) complex [39]. ATM, 53BP1 and MRN function to overcome the heterochromatic barrier to DSB repair [33] but the role of Artemis in the slow repair component has long remained enigmatic. Given that Artemis is a nuclease, the specific requirement for Artemis argues that the repair process underlying the slow component in G1 is enzymatically different to the fast repair process.

3.2. Artemis reveals the existence of a resection-dependent NHEJ process

A recent finding has shed light on the role of Artemis and provided evidence that NHEJ encompasses a process involving DSB end-resection. Although inactivation of the resection factor CtIP by siRNA did not affect DSB repair kinetics in wild-type G1 cells, it unexpectedly rescued the repair defect normally observed in Artemis-defective cells [40]. That is, Artemis mutants, which normally fail to repair DSBs with slow kinetics and exhibit unrepaired DSBs over many days [38,39], exhibit two-component repair kinetics indistinguishable to wild-type cells after CtIP inactivation [40]. This is reminiscent of the situation in G2 phase where CtIP siRNA rescued the repair defect in cells defective in downstream HR factors such as BRCA2, enabling the slowly repairing DSBs to be handled by NHEJ instead of HR [31]. Thus, the CtIP/Artemis result in G1 suggests that (i) Artemis functions down-stream of CtIP and (ii) in the absence of CtIP in G1 slowly repairing DSBs are handled by an Artemis-independent NHEJ process. Since the same result was obtained after inactivation of other known resection factors (e.g. MRE11 and EXO1), a model arose that the slow DSB repair process in G1 represents a resection-dependent NHEJ process during which Artemis resolves intermediate structures that arise after the initiation of resection [34,40]. Significantly, this uncovered resection-dependent NHEJ process functions in wild-type cells and involves the c-NHEJ factor DNA-PK [39,40]. Thus, these resected DSBs are repaired by c-NHEJ and not alt-NHEJ. To highlight this difference as well as the central role of Artemis in this repair pathway, we have named this process resection-dependent c-NHEJ or Artemis-dependent c-NHEJ [34].

Resection in G1 appears to be much shorter in length than in G2 since RPA foci are not routinely observed, consistent with the notion that extended resection to drive HR is not required. This raises the question of how resection occurs and its overlap with the processes in S/G2. A first clue was provided by the observation that MRE11 endonuclease, which initiates resection for HR by incising the DNA 'behind' Ku [37], is dispensable for DSB repair in G1 [40]. Thus, the mode of initiation of resection discussed above for two-ended DSBs during HR and replication-dependent seDSBs does not appear to be exploited in G1. Instead, MRE11 operates during resection-dependent c-NHEJ exclusively as an exonuclease together with EXO1. In contrast to the situation in G2 phase, Ku remains associated at the DSB during the slow repair process. As a working hypothesis, it was proposed that Ku

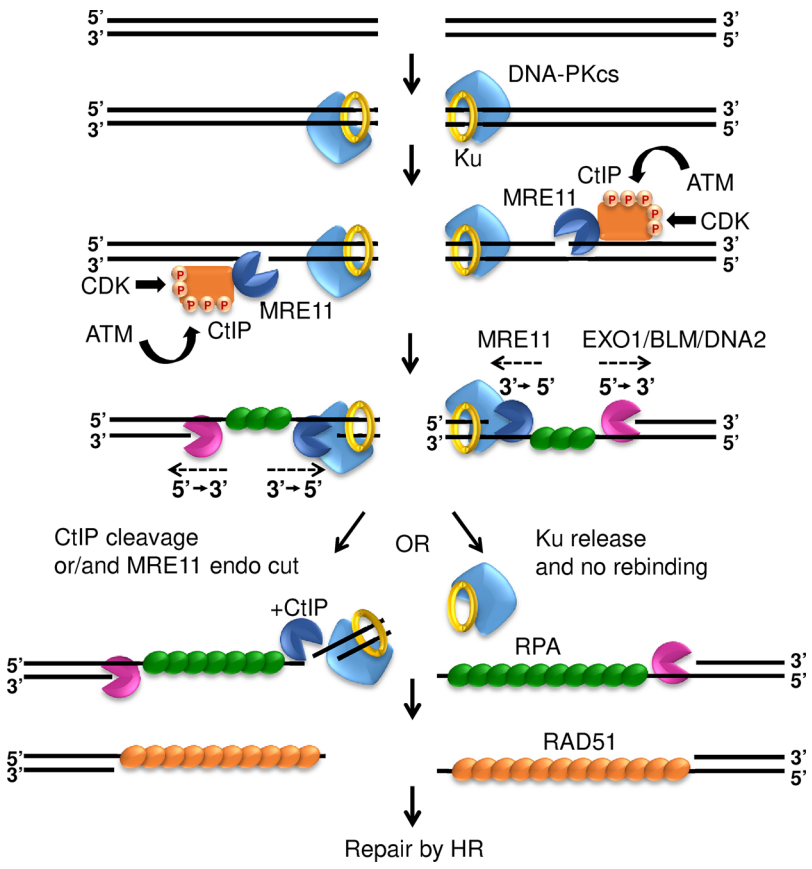

Fig. 3. Progression of resection at radiation-induced two-ended DSBs in G2 phase.
Ku and DNA-PKcs bind to DSBs and NHEJ ensues at many DSBs. At a subset of DSBs, resection is initiated by MRE11 endonuclease activity, which requires direct phosphorylation of CtIP at Ser664 and Ser745 by ATM as well as CtIP phosphorylation by CDK2 at Thr847 and Ser327. Bidirectional exonuclease activity ensues as shown in Fig. 1. The precise mechanism leading to Ku release has not been substantiated.

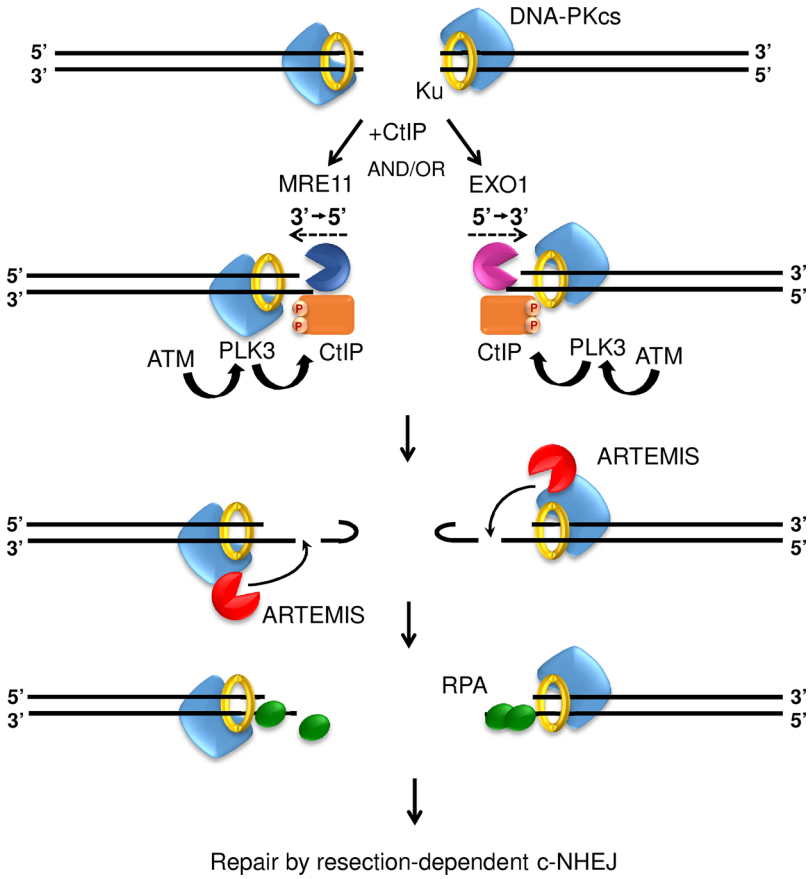

Fig. 4. Progression of resection at radiation-induced two-ended DSBs in G1 phase.
Ku and DNA-PKcs bind to all DSBs and 80% of the DSBs are repaired with fast kinetics by c-NHEJ. A subset of DSBs undergo repair by resection-dependent c-NHEJ. This process does not involve MRE11 endonuclease activity but does require PLK3-dependent phosphorylation of CtIP at Thr847 and Ser327. It is proposed that Ku translocates inwards allowing short regions of resection by the exonucleases shown. Artemis is required to remove a lesion created downstream of the exonucleases, which we propose could represent a hairpin-like structure. This process does not require phosphorylation of CtIP at Ser664 and Ser745 (our unpublished findings).

translocates inwards away from the end, a feature previously observed in biochemical assays [41], allowing the exonucleases access to the DSB for limited resection (Fig. 4). Another possibility is that the exonucleases 'push' Ku inwards during the resection process exposing the ends for further resection. This will generate DSB ends with 3′ or 5′ overhangs which, in biochemical assays, have been shown to require DNA-PKcs and Artemis for repair [42]. It has also been proposed that the short ss ends are captured by DNA-PKcs creating a hairpin-like structure that necessitates Artemis for cleavage [40]. Interestingly, cellular studies have recently shown that Artemis is required for the end-joining of DSBs with ss overhangs that arise in cells without BRCA2 [43]. Significantly, whilst resection in S/G2 is regulated by CDK phosphorylation of CtIP, in G1 the process is regulated by polo-like kinase 3 (PLK3). This and further features of resection in G1 are presented in Fig. 4 and will be discussed below.

4. Processing of blocked DSB ends prior to NHEJ

We initiated our evaluation of the processing of Ku-bound DNA ends by considering the analysis of Spo11-bound DSBs induced during meiosis. Here, we add to our analysis another class of blocked DSB end, namely DSBs arising during TOP2-mediated DNA unwinding [2]. TOP2 creates a transient DSB intermediate, a "cleavable complex", with the enzyme covalently linked to the 5'-termini by 5'-phosphotyrosyl bonds. Normally, TOP2 reseals the ends after completing its catalytic unwinding. Occasionally, however, the cleavable complex becomes abortive, forming a blocked DSB end. When this situation arises in G1 phase, extended resection as in S/G2 could be deleterious since a sister chromatid is not available to progress HR. Moreover, resection from the end as for resection-dependent c-NHEJ is precluded due to the covalent linkage between TOP2 and DNA. However, removal of the blocked lesion must take place to allow c-NHEJ and ideally occurs with minimal or no sequence loss. Since this is a common source of endogenous DNA breakage, 5'-tyrosyl DNA phosphodiesterase 2 (TDP2) has evolved to hydrolytically remove the trapped topoisomerase peptide [44]. This highly efficient process then couples to c-NHEJ without deletion formation. An alternative pathway involving proteosomal degradation also appears able to remove the blocked lesion with subsequent rejoining by c-NHEJ [2]. However, there is evidence for a further pathway with parallels to the processes discussed above at ends "blocked" by Ku-binding. This process was revealed in G0/G1 cells following treatment with etoposide, a drug which targets TOP2 and enhances the formation of TOP2-blocked DSBs [45,46]. Strikingly, MRE11 or NBS1 deficiency in patient cells, or CtIP depletion, impairs the ability to repair such DSBs. Thus, intriguingly, this back-up process has similarities, although also distinctions, to the processes discussed above (Fig. 5).

5. Similarities and distinctions in the processing of "blocked" lesions

Given the abundance of Ku and its powerful end-binding activity, it can be considered as a "blocking" DNA end lesion even though it binds DNA ends non-covalently and potentially transiently. Above we describe situations where "blocking" lesions, including covalently bound proteins and non-covalently bound Ku, have to be removed to promote repair by c-NHEJ, resection-dependent c-NHEJ or HR. Here we focus on the similarities and distinctions between these processes, considering how the distinctions are suited for the situation.

Arguably, the most interesting comparison is between the processes activating resection-dependent c-NHEJ in G1 versus HR in S/G2 phases. Two important criteria are required for resection-dependent c-NHEJ in G1. Firstly, Ku must remain or re-associate at the DSBs. Indeed, as noted above, Ku is maintained at the break site during resection in G1 whereas it is lost from DSBs at later times in S/G2 [40]. Additionally, slow repair in G1 can be stalled at any time by addition of a DNA-PK

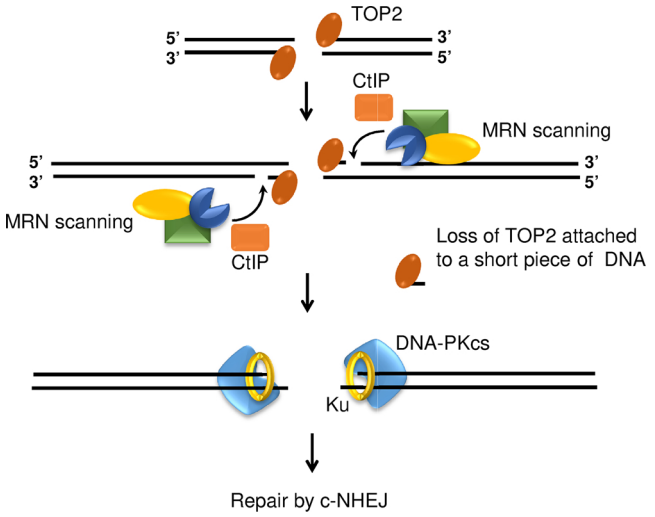

Fig. 5. Progression of NHEJ at DSBs generated by TOP2 cleavage.
TOP2 creates protein bridged DSBs similar to those generated by Spo11. TOP2 can be removed by TDP2 but there is evidence for an alternative pathway as depicted. This involves MRE11 endonuclease function activated by CtIP. The precise step leading to removal of TOP2 is unclear. It may arise simply from the upstream cleavage step or could involve a second cleavage by MRE11.

inhibitor. Since the catalytic function of DNA-PKcs depends on its binding to DNA-bound Ku, this suggests that DNA-PK must be present at the break site throughout the slow repair process. Secondly, the length of resection is necessarily shorter. Using a reporter construct with two closely located DSBs, we have estimated that resection events in G1 involve less than 20 nucleotides although longer patches can occur at α-particle induced DSBs and at events leading to mis-rejoining of ends from two more spatially separated DSBs [40].

5.1. Initiation of resection by MRE11 nuclease

In this context, it is striking that resection in S/G2 is initiated by MRE11 endonuclease activity internally to the DSB end whilst in G1 phase, the exonucleases initiate the process, with Ku remaining associated with the end [40]. This distinction provides an efficient route to rapidly generate a large length of ss-DNA when HR is utilised and to restrict resection when c-NHEJ ensues. This then raises the question of how TOP2-bound lesions in G1 are processed (when TDP2 is absent). Although the requirement for MRE11 endonuclease was not examined, a study involving single molecule imaging provided evidence that MRN can scan for DSB ends on nucleosome-coated DNA and when an end is found, it can effect an endonucleolytic incision [47]. Given the requirement for MRN in the back-up repair of blocked TOP2 lesions in G1, it is likely that this process also involves MRE11 endonucleolytic cleavage, even though subsequent rejoining involves Ku binding and repair by c-NHEJ. If this occurs, it argues that MRE11 endonuclease incision can arise in G1 but is specifically precluded during resection-dependent c-NHEJ. Significantly, the back-up process repairing TOP2-bound DSBs in G1 is likely to generate small deletions.

5.2. Ku removal from resected ends

Intimately connected to the step initiating resection is the downstream step removing the "blocked" lesion. As mentioned above, Ku must be removed and re-binding precluded to progress HR but it should beneficially remain associated at the DSB for c-NHEJ in G1. There are several ways that Ku could be removed in S/G2: i) by a second MRE11 endonucleolytic incision, ii) by CtIP flap endonuclease function, iii) by MRE11 exonuclease "pushing" Ku from the end since it is non-covalently bound, iv) by Ku phosphorylation reducing its affinity for DSB

ends [48], or v) by the inability of Ku to rebind during a dynamic release-binding process as the resection length increases. The last three possibilities most likely cannot take place at a lesion with a covalently bound protein. Single-molecule analysis of MRN activities at a blocked end provided evidence for a second MRE11 endonucleolytic event [47]. Of relevance in this context, the endonucleolytic activity of CtIP has been reported to require ATM-dependent phosphorylation of CtIP at Ser664 and Ser745 and is abolished in N289A/H290A CtIP mutants [28]. Significantly, efficient repair of, and Ku loss at, CPT-induced DSBs in S phase requires ATM and is abolished in N289A/H290A CtIP mutants, implicating the requirement for CtIP endonuclease activity [24]. Whether CtIP endonuclease activity is required for Ku removal during HR in G2 phase is less clear. Mutation of these phosphorylation sites impaired HR of IR-induced DSBs [28,31] although it was dispensable for restriction enzyme-induced DSB repair by HR [28]. In contrast, CtIP function in G1 occurs independently of ATM phosphorylation at Ser664 and Ser745, consistent with the notion that Ku is not removed in this situation where c-NHEJ progresses (unpublished data). CtIP endonuclease activity has also been suggested to be required for the back-up repair of TOP2-induced DSBs [28].

5.3. Activation of CtIP by upstream kinases

A second area of comparison involves the upstream kinases regulating the processes. Upon entry into cell cycle phases permissive for HR (S and G2 phases), CtIP undergoes phosphorylation at Thr847 by CDK2, which is important for the activation of MRE11 nuclease activity [49,50]. Additionally, CDK2-dependent phosphorylation at Ser327 mediates complex formation between CtIP and BRCA1, which is important for removing 53BP1, which is inhibitory for resection [51,52]. CDK2 is inactive in G1, and CtIP is activated after DSB induction by PLK3. PLK3 binds to phosphorylated CtIP via its polo box domain and mediates robust CtIP phosphorylation on Ser327 and Thr847 in a self-amplification process [53]. PLK3-dependent CtIP phosphorylation at Ser327 and Thr847 is required for resection-dependent c-NHEJ [40,53]. Additionally, CtIP phosphorylation on Thr847 but not on S327 is required for repairing TOP2-blocked lesions in G1 phase, suggesting that BRCA1 binding and 53BP1 removal might be less important for the repair of TOP2-blocked lesions than for DSBs undergoing resection-dependent c-NHEJ [45]. In summary, although the same two sites on CtIP are phosphorylated in G1 and S/G2, the upstream kinases are distinct, which imparts some further distinctions. Importantly, in S/G2 phase, CDK2 is constitutively active and CtIP is phosphorylated at Ser327 and Thr847 even in undamaged cells. In G1 phase, PLK3 is activated in a damage-dependent manner by ATM, and CtIP phosphorylation occurs 1–2 h after damage induction [40,53]. The more rapid activation of CtIP in G2 phase could be required for the more robust progression of resection compared to resection-dependent c-NHEJ.

5.4. Roles for BRCA1 and 53BP1

A further event required for resection is BRCA1-dependent relief of a barrier posed by 53BP1. In G2, this is promoted by CDK-dependent phosphorylation at CtIP Ser327, which mediates complex formation with BRCA1 [51,52], although the role of this CtIP modification during HR has been questioned [54,55]. BRCA1 is also required for HR following replication fork stalling/collapse. Relief of the 53BP1-dependent barrier can be visualised by enlargement of 53BP1 foci and creation of a 53BP1-devoid core reflecting repositioning of 53BP1 [56,57]. Significantly, BRCA1 relief of a 53BP1 barrier is also required for resection-dependent c-NHEJ in G1 although overt repositioning has not been visualised perhaps due to the shorter length of resection [40]. This important role for BRCA1 in resection-dependent c-NHEJ extends its function beyond promoting HR and provides a further connection between the processes interfacing Ku loss and resection.

6. Concluding remarks

A focus of many past and present studies is the mechanism underlying the choice between HR and NHEJ for repairing DSBs. Additionally, it has been widely assumed that Ku was precluded from binding seDSBs that arise following replication fork stalling/collapse. However, the abundance of Ku has increased from lower to higher organisms, and is substantially higher in primates compared to rodents. This high abundance coupled with its exquisite end-binding capacity has made Ku a formidable force in the race to bind DSB ends. We discuss recent insight into mechanisms that function to promote the eviction of Ku at DSBs when non-NHEJ pathways have advantages over c-NHEJ usage. Intriguingly, in some situations covalently bound proteins arise at DSBs and removal of these “blocking” lesions has parallels to the processes removing Ku. Collectively, these studies reveal that Ku plays a dynamic role at all DSB ends and participates in the final decision making process. The process necessitates a plethora of nucleases, which interplay in different ways. Many questions remain to be addressed including the precise role played by CtIP, the details leading to Ku eviction, when exactly resection is initiated in the distinct cell cycle phases and the signals that determine whether repair occurs without resection by c-NHEJ or whether the more complex route to DSB repair involving resection is traversed.

Conflict of interest

The authors declare no conflict of interest.

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